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Evaluating the Efficacy of Liquid Organic Fertilizers and Biofertilizers to Diminish the Mineral Nitrogen Doses for Spinach Plants

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TO MITIGATE the adverse effects of excessive mineral nitrogen fertilization on vegetative crops, it is imperative to decrease the doses of mineral nitrogen applied and explore alternative nitrogen sources to fulfill the plants' requirements. Therefore, a field experiment was conducted over two consecutive seasons (2022 and 2023) to examine the impact of bio fertilizer (either with seed inoculation with *Azospirillum brasilense* or without inoculation at 8 kg ha^{-1}), as main factor, on the growth and productivity of spinach plants. The plants were subjected to varying doses of mineral nitrogen (25%, 50%, 75%, and 100% of the Nitrogen Recommended Dose NRD) as sub main treatments, while different liquid organic fertilizers [none (control), compost tea at rate of 48 L ha^{-1}, liquid biogas at rate of 48 L ha^{-1}and vermicompost extract at rate of 48 L ha^{-1}]were evaluated as sub-sub treatments. Regarding bio fertilization, the results revealed that all growth and productivity parameters, including yield (Mg ha^{-1}), plant height (cm), and No. of leaves plant^{-1} as well as the chemical composition of leaves, (N, P, K %) attained their peak values when treated with *Azospirillum brasilense* inoculation compared to untreated plants. For example, yield of plants treated with bio fertilization were 24.51 and 25.92 Mg ha^{-1} for 1st and 2nd seasons, respectively,while the yield of the plants grown without bio fertilization were 21.18 and 22.66 Mg ha^{-1} for 1st and 2nd seasons, respectively. On the other hand, as the nitrogen recommended dose (NRD) decreased, the values of these parameters declined. Among the liquid organic fertilizers studied, biogas emerged as the most effective (yield values of 24.79 and 26.8 Mg ha^{-1} for 1st and 2nd seasons, respectively), followed by vermicompost extract and then compost tea, all surpassing the control (yield values of 20.03 and 21.61 Mg ha^{-1} for 1st and 2nd seasons, respectively). Importantly, the presence of bio fertilizer alongside any of the liquid organic fertilizers under 75% NRD resulted in higher values for growth and productivity parameters compared to plants fertilized with 100% NRD without bio fertilizer and liquid organic fertilizer. Concerning soil properties, the interaction among treatments did not have a significant effect on the studied properties. However, there was a noticeable relative improvement in fertility parameters due to the application of both bio fertilizer and liquid organic fertilizer. Finally, it is recommended to incorporate *Azospirillum brasilense* inoculation and biogas, vermicompost extract, or compost tea as alternatives to mineral nitrogen fertilization, especially at lower NRD levels, to optimize spinach production while maintaining soil fertility.

Keywords: *Azospirillum brasilense*, Compost tea, Biogas, Vermicompost extract.

1. Introduction

Spinach (*Spinacia oleracea*), renowned for its nutritional richness and economic significance in Egypt, holds a pivotal role in local diets and agricultural economics (**Ghoneam *et al.* 2022)**. Nitrogen plays a crucial role in the growth and

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development of spinach plants. However, the widespread use of excessive mineral nitrogen fertilization poses serious threats, not only to crop health but also to human well-being due to potential health hazards associated with nitrate accumulation in food. Recognizing the imperative of mitigating such risks, it becomes essential to emphasize the detrimental effects of over-reliance on mineral nitrogen fertilization. (**Ahmed *et al.* 2020, Alkharpotly *et al.* 2023, Farouk *et al.* 2023, Abd El-Hady *et al.* 2024)**.

This underscores the crucial role that biofertilization, particularly with nitrogen-fixing bacteria can play in alleviating dependence on mineral fertilizers (**Hindersah *et al.* 2020; Sumbul *et al*. 2020; Elzemrany and Faiyad 2021).** *Azospirillum brasilense* plays a significant role in nitrogen fixation. Indeed, *Azospirillum brasilense* is a bacterium known for its ability to fix nitrogen. It's a gram-negative, motile, and rod-shaped bacterium that forms part of the plant growth-promoting rhizobacteria (PGPR) group **(Galindo *et al.* 2020).** Nitrogen fixation is a crucial biological process where atmospheric nitrogen (N_2) is converted into ammonia (NH_3) or other nitrogen compounds usable by plants **Sarhan and Bashandy 2021).** This process is vital for the growth and development of plants because nitrogen is an essential element for their metabolic processes and overall growth. By associating with the roots of certain plants, such as cereals and grasses, *Azospirillum brasilense* can enhance nitrogen fixation and subsequently improve plant growth and yield **(Galindo *et al.* 2022)**.The relationship between *Azospirillum brasilense* and plants is often termed as a symbiotic relationship, where both parties benefit. The bacterium receives nutrients and a suitable environment for growth from the plant, while it provides the plant with fixed nitrogen, thereby reducing its dependence on synthetic nitrogen fertilizers **(Rondina *et al.* 2020).**Overall, *Azospirillum brasilense* and similar nitrogen-fixing bacteria play a significant role in agricultural practices aimed at reducing the need for chemical fertilizers, promoting sustainable farming, and improving soil health **(Zaheer *et al.* 2022)**.

Additionally, the adoption of organic fertilization methods such as biogas, compost tea, and vermicompost extract presents promising avenues to reduce reliance on mineral fertilization while concurrently enriching plant and soil health. Biogas, a byproduct of anaerobic digestion, offers benefits for both plant growth and soil fertility **(Ai *et al.* 2020; Møller *et al.* 2022; Farid *et al.* 2023).** Compost tea, a liquid fertilizer derived from compost, provides a rich source of nutrients and beneficial microorganisms to support plant and soil health (**Nada *et al.* 2023; Hafez *et al.* 2024)**. Similarly, vermicompost extract, a liquid extract of worm castings, serves as a potent organic fertilizer, enriching soil with essential nutrients and beneficial microorganisms (**Yusof *et al.* 2018; Abd El-Hady *et al*. 2021; Tikoria *et al.* 2022)**.

Thus, this study aims to investigate the potential of these alternative fertilization methods in optimizing spinach production while minimizing reliance on mineral nitrogen fertilization, thereby promoting sustainable agricultural practices and ensuring food security.

2. Material and Methods

- Experimental location

This work research was carried out on the Farm of Experimental Research Station of agricultural research institute in Sakha, Kafr El-Sheikh Governorate, Egypt (31^o 6' N latitude, 30^o 56' E longitude) during two consecutive seasons (2022 and 2023).

- Initial soil sample analyses

Before the sowing process, the initial soil samples were collected for analyzing using standard methods depending on **Sparks *et al.* (2020) and Dane and Topp (2020)** at a depth of 0-25 cm**.** Table 1 displays the fundamental soil characteristics before cultivation during seasons of 2022 and 2023.

- Experimental design and treatments

A field experiment was conducted to examine the impact of bio fertilizer (either with seed inoculation with *Azospirillum brasilense* or without inoculation at 8 kg ha^{-1}), as main factor, on the growth and productivity of spinach plants. The plants were subjected to varying doses of mineral nitrogen (25%, 50%, 75%, and

100% of the Nitrogen Recommended Dose NRD) as sub main treatments, while different liquid organic fertilizers [none (control), compost tea at rate of 48 L ha^{-1}, liquid biogas at rate of 48 L $ha^{-1}$and vermicompost extract at rate of 48 L ha^{-1}] were evaluated as sub-sub treatments.

- Studied bio and organic fertilizers

Azospirillum brasilense was obtained from the Microbiology Department, Sakha Agriculture Research Station, Agricultural Research Center, Egypt. The bacterium was grown in liquid Nutrient Broth medium with the following composition per liter: beef Extract 1.0g, peptone 5.0g, Yeast Extract 2.0g, Sodium Chloride 5.0g, with a final pH adjusted to 6.8 ± 0.2, and incubated at 28°C. Pure isolates of *Azospirillum brasilense* were grown in 500 ml flasks containing 250 ml of nutrient broth on a rotary shaker incubator at 28°C for 8 hours daily. After 3 days of inoculation, peat-based cultures of *Azospirillum brasilense* were prepared following the method described by **Difco (1985).** Cell suspensions containing 10^7 cfu mL^{-1} were used to impregnate sterilized peat at a rate of 52 ml liquid culture per 100 grams of peat. The inoculated peat was thoroughly mixed and allowed to mature at room temperature for 48 hours.

Different liquid organic fertilizers, including compost tea, liquid biogas and vermicompost extract were procured from the Egyptian commercial market, Corn Company for Sustainable Development, and their characteristics are shown in Table 2.

- Cultivation and harvest

Seeds of spinach (cv. Balady) were treated with bio fertilizer according to the studied treatments, as seeds were coated with a 10% solution of Arabic gum in water, acting as an adhesive material **(Hamdi, 1982),** before inoculation with the *Azospirillum brasilense* peat-based preparation. After coating, the seeds were air-dried in the shade for 30 minutes and then promptly sown. Seeds were sown in rows at a rate of 28.8 kg ha^{-1} on the first week of September during both studied seasons. The sub sub-main plot area was 6.0 m^2 (2.0 x 3.0 m). The recommended dose of mineral nitrogen for spinach production is 60 units of nitrogen (N) per feddan according to the MASR, taking into account the available nitrogen content of the soil (30 mg kg^{-1}). The treatments of mineral nitrogen were added according to the studied treatments as ammonium sulphate (21%N). Different liquid organic fertilizers were added according to the studied treatments with the first and second irrigation events. Additionally, all plots received 28.8 units of phosphorus (P) per hectare as calcium superphosphate (6.7% P) during soil preparation, and 60 units of potassium (K) per hectare as potassium sulfate (39.8% K) with the second irrigation event. Irrigation was performed every 10 days. Spinach was harvested during the pre-flowering stage, precisely when it attained the 5-6 leaf growth stage, which occurred approximately 60 days after sowing.

- Measurements

Yield (Mg ha^{-1}), plant height (cm) and No. of leaves $plant^{-1}$ were measured at harvest. Also, the chemical composition of leaves (N, P, K %) were determined. Plant samples digestion was done using $HClO_4$ + H_2SO_4 as mixture (**Peterburgski 1968**). Then N, P, and K percentages were determined using Micro-Kjeldahl, spectrophotometric and flame photometer, respectively (**Walinga *et al.* 2013).** Also, nitrogen uptake (kg ha^{-1}) was measured (N concentration in leaves X dry weight). Nitrate and nitrite (mg kg^{-1}) contents in spinach leaves at harvest stage were determined as described by **Ozdestan and Uren (2010).** Additionally, soil properties were evaluated at the harvest stage, encompassing bulk density (Mg m^{-3}), total porosity (%), pH, electrical conductivity (EC, dSm^{-1}), organic matter content (%) and available nitrogen (mg kg^{-1}) according to **Sparks *et al.* (2020) and Dane and Topp (2020)**. The total microbial count (CFU mL^{-1}) in the soil was determined by estimating the total count of microorganisms in the plant Rhizosphere soils, following the method described by **Allen (1959).**

- Statistical analyses

Statistical analysis was done as described by **Gomez and Gomez (1984)** *via* **CoStat (Version 6.303, CoHort, USA, 1998-2004)** and DMRT (Duncan's Multiple Range Test)**.**

Table 1. Fundamental soil characteristics before cultivation during seasons of 2022 and 2023.

Characteristics	Values		Characteristics	Values	
	1st season	2nd season		1st season	2nd season
Chemical traits			**Availability of nutrients**		
O.M	1.21,%	1.23,%	**Available -N**	30.0, mg kg^{-1}	30.12, mg kg^{-1}
EC	3.75, dSm^{-1}	3.34, dS m^{-1}	**Available -P**	9.52, mg kg^{-1}	9.43, mg kg^{-1}
pH	8.27	8.26	**Available -K**	238, mg kg^{-1}	242, mg kg^{-1}
Particle size distribution			**Physical properties**		
			Bulk density	1.42, Mg m^{-3}	1.41, Mg m^{-3}
Sand	18.65,%	17.91,%	**Total porosity**	46.42,**%**	46.79,**%**
Silt	29.53,%	29.46,%	**Hydro physical properties**		
Clay	51.82,%	52.63,%	**F.C**	42.12,%	41.85,%
Texture class	**Clay**		**W.P**	20.42,%	21.94,%

Table 2. Characteristics of the liquid organic fertilizers.

Biogas fertilizer		
Total potassium humate,%	2.10	
Humic acid,%	1.60	
Fulvic acid,%	0.35	
pH	7.52	
EC,dSm^{-1}	13.69	
N,%	1.50	
P,%	0.50	
K,%	0.20	
Vermicompost extract (VC) and Compost tea (CT)		
	VC	**CT**
pH	8.45	8.30
EC,dS m^{-1}	10.2	9.97
Fe, mg kg^{-1}	0.90	1.00
Mn, mg kg^{-1}	18.95	20.0
Zn, mg kg^{-1}	19.15	22.0
B, mg kg^{-1}	27.6	31.3
Cu, mg kg^{-1}	45.9	46.2

3. Results

Spinach yield and vegetative traits

Table 3 shows the individual effect of bio fertilization and various organic sources on yield (Mg ha^{-1}), plant height (cm) and No. of leaves plant^{-1} of spinach plants fertilized with diverse mineral nitrogen dosages across 2022 and 2023 seasons. While Table 4 illustrates the interaction effect among the studied treatments during seasons of 2022 and 2023 seasons. Regarding bio fertilization, the Table 3 reveals that yield (Mg ha^{-1}), plant height (cm) and No. of leaves plant^{-1} attained their peak values when treated with *Azospirillum brasilense* inoculation compared to untreated plants. On the other hand, as the nitrogen recommended dose (NRD) decreased, the values of these parameters declined (Table 3). Among the liquid organic fertilizers studied, biogas emerged as the most effective, followed by vermicompost extract and then compost tea, all surpassing the control (Table 3). Regarding interactive effects, the highest values of yield (Mg ha^{-1}), plant height (cm) and No. of leaves plant^{-1} were attained with the combined treatment denoted as (**B_1x N_1x L_3**) (Table 4). Importantly, the presence of bio fertilizer alongside any of the liquid organic fertilizers under 75% NRD resulted in higher values for yield (Mg ha^{-1}), plant height (cm) and No. of leaves plant^{-1} compared to plants fertilized with 100% NRD without bio fertilizer and liquid organic fertilizer. The same trend was found in both seasons.

Table 3. Impact of bio fertilization and various organic sources on yield and components of spinach plants fertilized with diverse mineral nitrogen dosages across 2022 and 2023 seasons: Individual effect of the studied treatments.

Treatments	Yield, Mg ha^{-1}		Plant height, cm		No. leaves plant^{-1}	
	1st season	2nd season	1st season	2nd season	1st season	2nd season
Main treatments (bio fertilization)						
With bio fertilizer (B_1)	24.51 a	25.94 a	31.73 a	32.44 a	10.38 a	10.48 a
Without bio fertilizer (B_2)	21.18 b	22.66 b	31.52 b	32.02 b	10.30 b	10.38 a
LSD at 5%	0.129	0.178	0.108	0.136	0.0465	0.155
Sub main treatments (nitrogen recommended dose)						
100% of NRD (N_1)	28.45 a	30.96 a	33.11 a	34.02 a	10.92 a	11.09 a
75% of NRD (N_2)	26.39 b	27.83 b	32.48 b	32.85 b	10.70 b	10.75 b
50% of NRD (N_3)	21.35 c	22.55 c	30.73 c	31.37 c	10.38 c	10.44 c
25% of NRD (N_4)	15.17 d	15.86 d	30.19 d	30.67 d	9.36 d	9.45 d
LSD at 5%	0.065	0.0474	0.086	0.086	0.06	0.055
Sub-sub main treatments (organic fertilization)						
Control (L_1)	20.03 d	21.61 d	30.55 c	31.08 c	10.09 d	10.17 d
Compost tea (L_2)	22.46 c	23.62 c	31.85 b	32.41 b	10.28 c	10.36 c
Biogas (L_3)	24.79 a	26.80 a	32.24 a	33.04 a	10.58 a	10.7 a
Vermicompost (L_4)	24.09 b	25.18 b	31.87 b	32.4 b	10.41 b	10.50 b
LSD at 5%	0.056	0.049	0.073	0.09	0.0466	0.0549

Means within a column followed by a different letter (s) are statistically different at 5%

Table 4. Impact of bio fertilization and various organic sources on yield and components of spinach plants fertilized with diverse mineral nitrogen dosages across 2022 and 2023 seasons: Interaction effect of the studied treatments.

Treatments	Yield, Mg ha^{-1}		Plant height, cm		No. leaves plant^{-1}	
	1st season	2nd season	1st season	2nd season	1st season	2nd season
(B_1)(N_1)(L_1)	28.01 f	30.37 f	32.63g	33.23 g	10.8 bcd	10.83 de
(B_1)(N_1)(L_2)	28.84 d	32.88 c	33.3 bcd	34.6 c	11.0 a	11.06 bc
(B_1)(N_1)(L_3)	32.07 a	34.95 a	33.7 a	35.7 a	11.0 a	11.5 a
(B_1)(N_1)(L_4)	29.40 c	33.3 b	33.5 ab	34.93 b	11.0 a	11.36 a
(B_1)(N_2)(L_1)	23.92 n	26.90 k	31.06 j	31.32 m	10.56 fgh	10.63 fgh
(B_1)(N_2)(L_2)	26.35 j	28.34 i	32.83 fg	33.4 fg	10.7 def	11 bcd
(B_1)(N_2)(L_3)	29.35 c	31.27 e	33.16 de	33.66 ef	11 a	10.73 efg
(B_1)(N_2)(L_4)	27.67 g	29.54 g	33 ef	33.26 g	10.76 cd	10.76 efg
(B_1)(N_3)(L_1)	21.04 q	21.83 t	29.66 q	30.83 n	10.1 j	10.16 kl
(B_1)(N_3)(L_2)	23.89 n	23.98 q	31.16 j	31.86 k	10.3 i	10.36 ij
(B_1)(N_3)(L_3)	27.27 h	25.41 n	31.53 i	32.16 j	10.76 cd	10.83 de
(B_1)(N_3)(L_4)	25.55 l	24.44 p	31.06 j	31.46 lm	10.53 gh	10.6 gh
(B_1)(N_4)(L_1)	12.24 z	14.82 B	29.66 q	31.36 m	9.0 o	9.06 q
(B_1)(N_4)(L_2)	16.86 w	16.6 z	30.47 mn	30.66 no	9.3 m	9.4 op
(B_1)(N_4)(L_3)	20.6 r	20.4 v	30.83 kl	30.5 o	9.8 k	9.83 m
(B_1)(N_4)(L_4)	19.10 t	20.04 w	30.16 op	30.16 p	9.53 l	9.66 n
(B_2)(N_1)(L_1)	25.35 m	26.41 l	32.03 h	32.46 i	10.76 cd	10.86 de
(B_2)(N_1)(L_2)	26.59 i	27.86 j	33.03 ef	33.5 efg	11 a	11.13 b
(B_2)(N_1)(L_3)	30.16 b	32.45 d	33.46 bc	34.03 d	10.93 ab	11.06 bc
(B_2)(N_1)(L_4)	27.21 h	29.47 g	33.26 cd	33.76 e	10.86 abc	10.9 cde
(B_2)(N_2)(L_1)	23.01 o	24.91 o	31.03 jk	31.46 lm	10.46 gh	10.6 gh
(B_2)(N_2)(L_2)	26.04 k	26.16 m	32.63 g	32.86 h	10.6 efg	10.63 fgh
(B_2)(N_2)(L_3)	28.27 e	28.61 h	33.13 de	33.6 ef	10.8 bcd	10.86 de
(B_2)(N_2)(L_4)	26.55 i	26.95 k	33 ef	33.23 g	10.73 cde	10.8 ef
(B_2)(N_3)(L_1)	17.75 u	18.42 x	28.86 r	29.5 q	10.03 j	10.1 l
(B_2)(N_3)(L_2)	20.05 s	20.88 u	31.13 j	31.66 kl	10.23 i	10.26 jk
(B_2)(N_3)(L_3)	13.27 y	23.35 r	31.43 i	32.26 ij	10.7 def	10.73 efg
(B_2)(N_3)(L_4)	21.99 p	22.13 s	31 jk	31.26 m	10.43 h	10.5 hi
(B_2)(N_4)(L_1)	8.90 b	9.25 D	29.46 q	29.7 q	9 o	9.1 q
(B_2)(N_4)(L_2)	11.083 a	12.25 C	30.28 no	30.73 no	9.16 n	9.26 p
(B_2)(N_4)(L_3)	17.39 v	17.95 y	30.66 lm	31.53 lm	9.7 k	9.76 mn
(B_2)(N_4)(L_4)	15.23 x	15.54 A	30 p	30.76 no	9.43 l	9.5 o
LSD at 5%	0.158	0.139	0.208	0.255	0.131	0.155

Means within a column followed by a different letter (s) are statistically different at 5%

Leaves chemical constituents

Table 5 presents the individual effect of bio fertilization, mineral nitrogen doses and different organic sources on the chemical composition of leaves (nitrogen, phosphorus and potassium, %) and nitrogen uptake (kg ha^{-1}) of spinach plants across the 2022 and 2023 seasons. Additionally, Table 6 depicts the combined effects of the studied treatments during both seasons. Regarding bio fertilization, the Table 5 reveals that the highest values of leaves nitrogen, phosphorus, potassium (%) and nitrogen uptake (kg ha^{-1}) were recorded when plants treated with *Azospirillum brasilense* inoculation compared to untreated plants. On the other hand, the values of leaves nitrogen, phosphorus, potassium (%) and nitrogen uptake (kg ha^{-1}) decreased as the nitrogen recommended dose (NRD) decreased (Table 5). Concerning the liquid organic fertilizers studied, the highest values of leaves nitrogen, phosphorus, potassium (%) and nitrogen uptake (kg ha^{-1}) when spinach fertilized with biogas followed by that of plants fertilized with vermicompost extract then that of plants fertilized with compost tea, all surpassing the control (Table 5). Regarding interactive effects, the peak values of leaf nitrogen, phosphorus, potassium percentages and nitrogen uptake (kg ha^{-1}) were achieved through the combined treatment labelled as **($B_1xN_1xL_3$)** (Table 6). Notably, when bio fertilizer was present alongside any of the liquid organic fertilizers at 75% nitrogen recommended dosage (NRD), it led to higher values for leaf nitrogen, phosphorus, potassium percentages and nitrogen uptake (kg ha^{-1}) compared to plants fertilized with 100% NRD without bio fertilizer and liquid organic fertilizer. Similar patterns were observed in both seasons.

Table 5. Impact of bio fertilization and various organic sources on leaves chemical constituents of spinach plants fertilized with diverse mineral nitrogen dosages across 2022 and 2023 seasons: Individual effect of the studied treatments.

Treatments	N,%		N-uptake, kg ha^{-1}		P,%		K,%	
	1st season	2nd season	1st season	2nd season	1st season	2nd season	1st season	2nd season
Main treatments (bio fertilization)								
Bio fertilizer (B_1)	2.63 a	2.86 a	0.65 a	0.74a	0.636 a	0.712 a	1.920 a	2.11 a
without bio fertilizer (B_2)	2.29 b	2.43 b	0.49b	0.55b	0.609 a	0.621 b	1.765b	1.97 b
LSD at 5%	0.0161	0.03	0.0008	0.098	0.028	0.0148	0.015	0.013
Sub main treatments (nitrogen recommended dose)								
100% of NRD (N_1)	3.24 a	3.49 a	0.92a	1.08a	0.659 a	0.741 a	2.83 a	3.03 a
75% of NRD (N_2)	2.64 b	2.84 b	0.69b	0.79b2	0.636 b	0.67 b	1.95 b	2.15 b
50% of NRD (N_3)	2.10 c	2.27 c	0.45c	0.51c	0.609 c	0.647 c	1.40 c	1.60 c
25% of NRD (N_4)	1.85 d	1.97 d	0.28d	0.31d	0.585 d	0.607 d	1.17 d	1.37 d
LSD at 5%	0.0168	0.0243	0.001	0.069	0.012	0.0086	0.0136	0.017
Sub-sub main treatments (organic fertilization)								
Control (L_1)	2.09 d	2.242 d	0.42d	0.48d	0.398 d	0.420 d	1.57 d	1.77 d
Compost tea (L_2)	2.49 c	2.64 c	0.56c	0.62c	0.656 c	0.712 c	1.79 c	1.99 c
Biogas (L_3)	2.69 a	2.92 a	0.67a	0.78a	0.734 a	0.785 a	2.05 a	2.26 a
Vermicompost (L_4)	2.57 b	2.77 b	0.62b	0.70b	0.701 b	0.748 b	1.94 b	2.13 b
LSD at 5%	0.013	0.017	0.0008	0.0647	0.0088	0.0087	0.0178	0.0173

Means within a column followed by a different letter (s) are statistically different at 5%

Table 6. Impact of bio fertilization and various organic sources on leaves chemical constituents of spinach plants fertilized with diverse mineral nitrogen dosages across 2022 and 2023 seasons: Interaction effect of the studied treatments.

Treatments	N,%		N-uptake, kg ha^{-1}		P,%		K,%	
	1st season	2nd season	1st season	2nd season	1st season	2nd season	1st season	2nd season
$(B_1)(N_1)(L_1)$	3.20 d	3.45 d	0.89d	1.04cd	0.433 p	0.48 o	2.50 e	2.68 e
$(B_1)(N_1)(L_2)$	3.37 c	3.65 c	0.97 c	1.20c	0.723 def	0.89 b	2.92 c	3.13 c
$(B_1)(N_1)(L_3)$	3.53 a	3.93 a	1.13 a	1.37a	0.796 a	0.956 a	3.17 a	3.35 a
$(B_1)(N_1)(L_4)$	3.44 b	3.81 b	1.01b	1.26b	0.74 cd	0.936 a	3.06 b	3.23 b
$(B_1)(N_2)(L_1)$	2.62 i	2.80 i	0.62	0.75e	0.413 pq	0.43 pq	1.83 k	2.03 k
$(B_1)(N_2)(L_2)$	2.71 h	2.95 h	0.71ef	0.83de	0.68 hijk	0.726 ghi	1.97 ij	2.13 j
$(B_1)(N_2)(L_3)$	3.03 f	3.37 e	0.89d	1.05cd	0.753 bc	0.856 c	2.23 g	2.4 g
$(B_1)(N_2)(L_4)$	2.84 g	3.14 g	0.78e	0.93d	0.743 cd	0.78 e	2.15 h	2.34 h
$(B_1)(N_3)(L_1)$	2.21 p	2.33 n	0.46i	0.51h	0.403 qr	0.456 op	1.34 p	1.50 p
$(B_1)(N_3)(L_2)$	2.37 mn	2.55 l	0.56h	0.61g	0.65 lmn	0.716 hij	1.42 o	1.62 o
$(B_1)(N_3)(L_3)$	2.45 k	2.75 j	0.67g	0.70f	0.73 cde	0.81 d	1.6 lm	1.82 m
$(B_1)(N_3)(L_4)$	2.4 lm	2.64 k	0.61gh	0.65g	0.706 efgh	0.753 fg	1.56 mn	1.76 n
$(B_1)(N_4)(L_1)$	1.35 v	1.45 t	0.16l	0.21l	0.383 rs	0.43 pq	0.93 t	1.15 s
$(B_1)(N_4)(L_2)$	2.07 r	2.17 p	0.35k	0.36jk	0.633 n	0.676 kl	1.18 r	1.38 q
$(B_1)(N_4)(L_3)$	2.35 n	2.54 l	0.48i	0.52h	0.703 efgh	0.77 ef	1.54 n	1.72 n
$(B_1)(N_4)(L_4)$	2.17 q	2.25 o	0.41j	0.45i	0.686 ghij	0.733 gh	1.27 q	1.45 p
$(B_2)(N_1)(L_1)$	2.84 g	2.95 h	0.72ef	0.78ef	0.41 pqr	0.433 pq	2.35 f	2.52 f
$(B_2)(N_1)(L_2)$	3.14 e	3.31 f	0.83de	0.92d	0.69 ghij	0.72 hij	2.72 d	2.96 d
$(B_2)(N_1)(L_3)$	3.22 d	3.44 d	0.97c	1.17c	0.773 ab	0.783 e	3.01 b	3.23 b
$(B_2)(N_1)(L_4)$	3.19 d	3.36 e	0.87de	0.99d	0.71 efg	0.733 gh	2.91 c	3.14 c
$(B_2)(N_2)(L_1)$	2.30 o	2.45 m	0.53h	0.61g	0.383 rs	0.41 q	1.63 l	1.90 l
$(B_2)(N_2)(L_2)$	2.42 kl	2.46 m	0.63gh	0.64g	0.656 klmn	0.7 ijk	1.85 k	2.05 k
$(B_2)(N_2)(L_3)$	2.74 h	2.91 h	0.77e	0.83de	0.753 bc	0.74 gh	2.02 i	2.21 i
$(B_2)(N_2)(L_4)$	2.5 j	2.69 k	0.66g	0.72f	0.71 efg	0.716 hij	1.95 j	2.14 j
$(B_2)(N_3)(L_1)$	1.16 w	1.26 u	0.21l	0.23l	0.37 s	0.376 r	1.18 r	1.37 q
$(B_2)(N_3)(L_2)$	2.02 s	2.12 q	0.41ij	0.44i	0.64 mn	0.656 lm	1.27 q	1.46 p
$(B_2)(N_3)(L_3)$	2.16 q	2.29 no	0.27kl	0.53h	0.7 fghi	0.716 hij	1.44 o	1.66 o
$(B_2)(N_3)(L_4)$	2.08 r	2.26 o	0.46i	0.50h	0.673 ijkl	0.693 jk	1.45 o	1.62 o
$(B_2)(N_4)(L_1)$	1.07 x	1.23 u	0.10m	0.11m	0.393 qrs	0.346 s	0.78 u	0.97 t
$(B_2)(N_4)(L_2)$	1.84 u	1.94 s	0.20l	0.24l	0.58 o	0.613 n	1.04 s	1.23 r
$(B_2)(N_4)(L_3)$	2.02 s	2.16 pq	0.35k	0.39j	0.663 jklm	0.65 m	1.45 o	1.67 o
$(B_2)(N_4)(L_4)$	1.95 t	2.06 r	0.29kl	0.32k	0.64 mn	0.643 m	1.17 r	1.38 q
LSD at 5%	0.037	0.051	0.002	0.183	0.0249	0.0248	0.05	0.04

Means within a column followed by a different letter (s) are statistically different at 5%

Where: B1: With bio fertilizer; **B2:** Without biofertilizer; **N1:**100% of NRD; **N2:**75% of NRD,**N3:** 50% of NRD; **N4:** 25% of NRD; **L1:** Control; **L2:** Compost tea**; L3:** Biogas and **L4:**Vermicompost

Quality parameters (nitrate and nitrite)

Fig 1 displays the concentrations of nitrate and nitrite (NO_3-N and NO_2-N, measured in mg kg^{-1}) in spinach plant leaves influenced by bio fertilization treatments across both study seasons, while Fig 2 illustrates the impact of mineral nitrogen fertilization treatments on these concentrations. Furthermore, Fig 3 presents the nitrate and nitrite concentrations influenced by the various liquid organic fertilizers.

In Fig 1, it's evident that the presence of bio fertilizer led to a reduction in the accumulation of nitrate and nitrite (NO_3-N and NO_2-N, measured in mg kg^{-1}). Conversely, the accumulation of nitrate and nitrite increased with higher nitrogen-recommended doses (NRD) as depicted in Fig 2. Additionally, all examined liquid organic fertilizers resulted in decreased accumulation of nitrate and nitrite compared to the control treatment, with the lowest values observed with biogas usage (Fig 3).

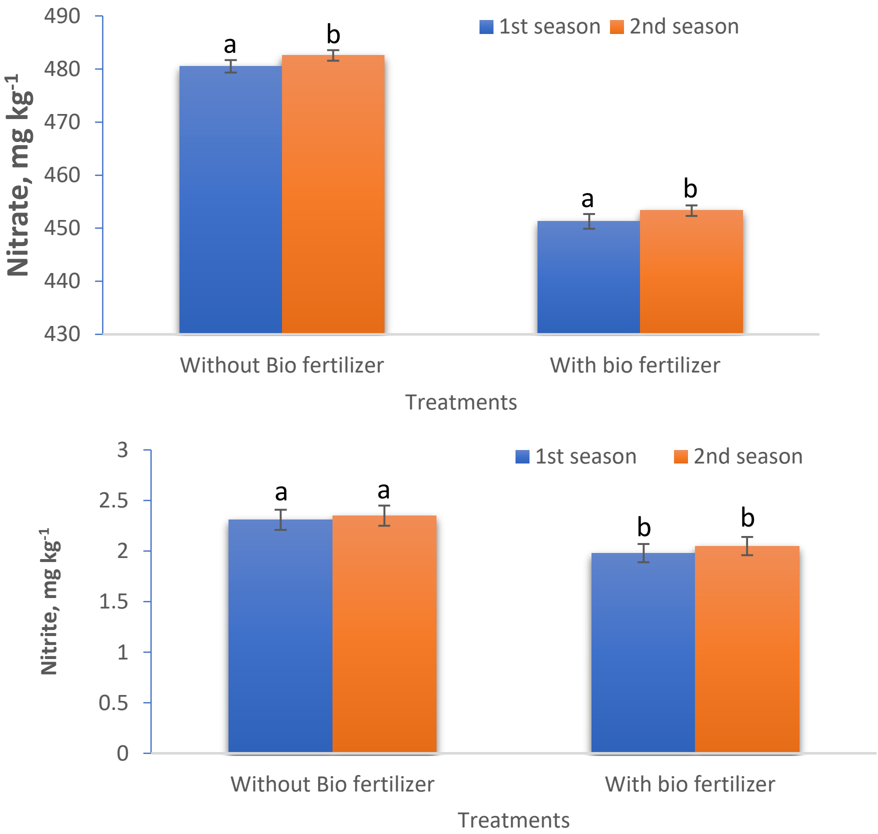


Fig. 1. The concentrations of nitrate and nitrite (mg kg^{-1}) in spinach plant leaves influenced by bio fertilization treatments across both study seasons.

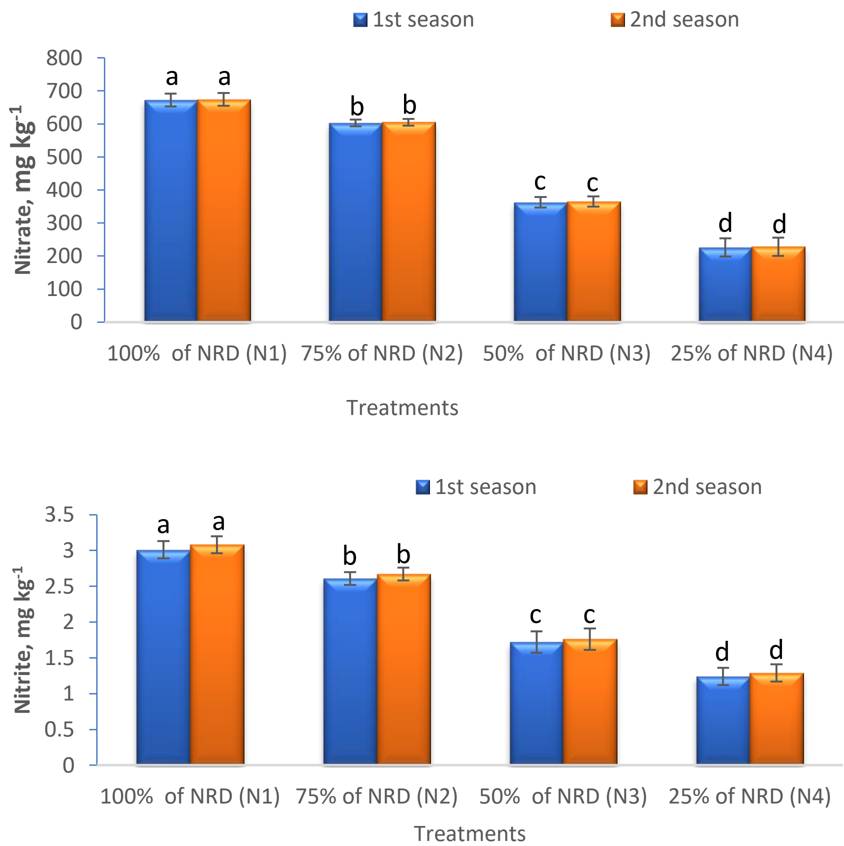
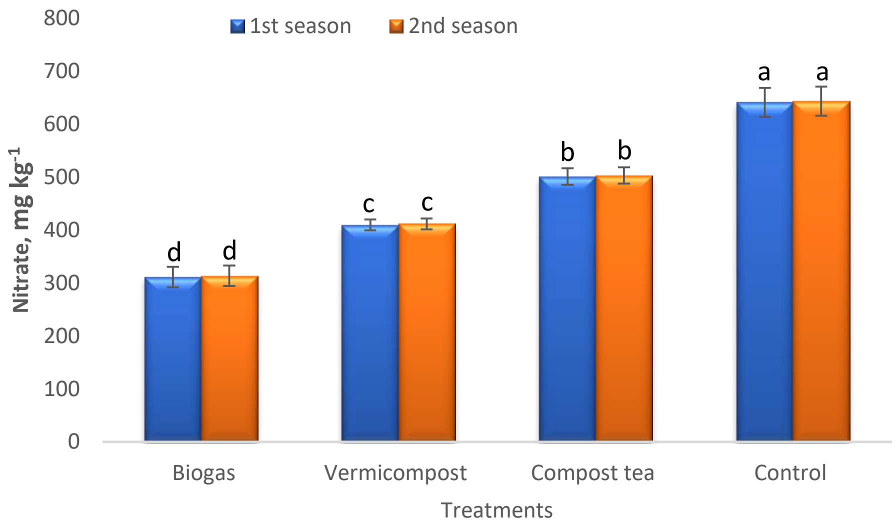


Fig. 2. The concentrations of nitrate and nitrite (mg kg^{-1}) in spinach plant leaves influenced by nitrogen fertilization treatments across both study seasons.



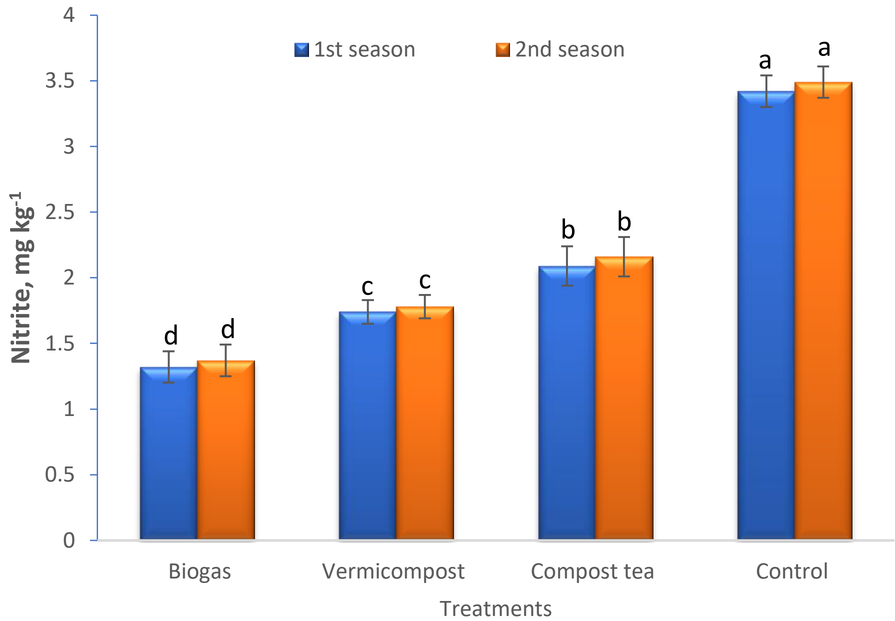


Fig. 3. The concentrations of nitrate and nitrite (mg kg^{-1}) in spinach plant leaves influenced by the various liquid organic fertilizers across both study seasons.

Postharvest soil analysis and total microbial count

Table 7 indicate the individual effects on soil properties, encompassing bulk density (Mg m^{-3}), total porosity (%), pH, electrical conductivity (EC, dSm^{-1}), organic matter content (%) and total microbial count during the seasons of 2022 and 2023. The interaction among treatments did not have a significant effect on the studied properties. However, there was a noticeable relative improvement in fertility parameters such as organic matter (%) due to the application of both bio fertilizer and liquid organic fertilizer. Fig 4 illustrate the effect of the studied treatments on the soil's available nitrogen levels (mg kg^{-1}). The utilization of bio fertilizer resulted in elevated levels of available nitrogen at the harvest stage compared to those in the soil samples that did not receive bio fertilizer. On the other hand, the soil's nitrogen availability increased with higher nitrogen doses. Concerning the liquid organic fertilizers, soils treated with biogas exhibited the highest available nitrogen values at the harvest stage, followed by those treated with vermicompost extract and then compost tea. Constant trends were noted across both seasons.

Two seasons of field experiments inoculated with nitrogen-fixing *Azospirillum brasilense* bacteria showed variations in the total microbial count. As documented in Table 7 inoculation with

Azospirillum brasilense increased the total microbial count (CFU mL^{-1}) in all treatments compared to the control (without inoculation). However, within the inoculation treatment itself, variations were observed. The Vermicompost treatment exhibited the highest total microbial count compared to other treatments, while the control showed the lowest count. The results were consistent in the second season, showing an increase in total microbial count compared to the first season.

Table 7. Impact of bio fertilization, diverse mineral nitrogen dosages and various organic sources on soil and rhizosphere properties across 2022 and 2023 seasons: Individual effect of the studied treatments.

Treatments	Bulk density (Mg m^{-3})		Total porosity (%)		PH		EC dSm^{-1}	
	1st season	2nd season	1st season	2nd season	1st season	2nd season	1st season	2nd season
Main treatments (bio fertilization)								
Bio fertilizer (B_1)	1.38 b	1.37 b	47.61 a	47.95 a	8.24 a	8.23 a	3.64 a	3.32 a
without bio fertilizer (B_2)	1.39 a	1.38 a	47.41b	47.70 b	8.25 a	8.24 a	3.65 a	3.33 a
LSD at 5%	0.0032	0.0047	0.121	0.178				
Sub main treatments (nitrogen recommended dose)								
100% of NRD (N_1)	1.37 d	1.37 c	47.98 a	48.30 a	8.23 a	8.22 a	3.61 b	3.31 c
75% of NRD (N_2)	1.38 c	1.37 b	47.65b	47.95 b	8.24 a	8.23 a	3.62 b	3.31bc
50% of NRD (N_3)	1.39 b	1.38 a	47.32 c	47.62 c	8.25a	8.24 a	3.67 a	3.33 ab
25% of NRD (N_4)	1.40 a	1.39 a	47.09 d	47.43 c	8.25a	8.24 a	3.68 a	3.34 a
LSD at 5%	0.0031	0.0055	0.117	0.208			0.0324	0.0199
Sub-sub main treatments (organic fertilization)								
Control (L_1)	1.41 a	1.41 a	46.68 d	46.98 d	8.25 a	8.25 a	3.71 a	3.34 a
Compost tea (L_2)	1.39 b	1.39 b	47.34 c	47.56 c	8.24 a	8.24 a	3.66 b	3.33ab
Biogas (L_3)	1.36 d	1.35 d	48.41 a	48.85 a	8.22 a	8.23 a	3.58 d	3.30 c
Vermicompost (L_4)	1.38 c	1.38 c	47.62 b	47.92 b	8.24 a	8.23 a	3.64 c	3.32 b
LSD at 5%	0.0052	0.0039	0.198	0.15			0.0176	0.0139

Treatments	Organic matter (%)		Total microbial (CFU mL^{-1})	
	1st season	2nd season	1st season	2nd season
Main treatments (bio fertilization)				
Bio fertilizer (B_1)	1.23 a	1.25 a	4.49 a	4.72 a
without bio fertilizer (B_2)	1.23 a	1.24 a	3.40 b	3.64 b
LSD at 5%	0.0062		0.169	0.284
Sub main treatments (nitrogen recommended dose)				
100% of NRD (N_1)	1.24 a	1.26 a	3.91 b	4.10 b
75% of NRD (N_2)	1.23 b	1.24 b	4.03 a	4.31 a
50% of NRD (N_3)	1.23 b	1.24 b	4.05 a	4.35 a
25% of NRD (N_4)	1.22 bc	1.24 b	3.79 c	3.97 c
LSD at 5%	0.0053	0.0044	0.107	0.114
Sub-sub main treatments (organic fertilization)				
Control (L_1)	1.21 c	1.22 c	3.78 c	4.05 c
Compost tea (L_2)	1.23 b	1.24 b	3.85 bc	4.06 c
Biogas (L_3)	1.25 a	1.27 a	3.99 b	4.23 b
Vermicompost (L_4)	1.23 b	1.24 b	4.17 a	4.39 a
LSD at 5%	0.005	0.0047	0.141	0.135

Means within a column followed by a different letter (s) are statistically different at 5%

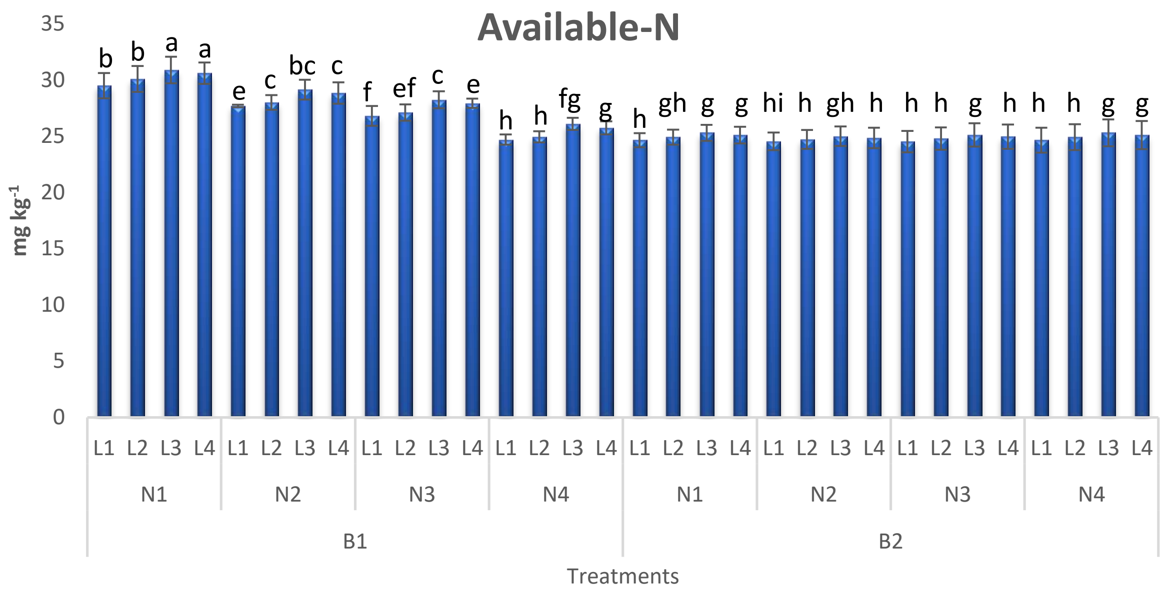


Fig. 4. Impact of bio fertilization, diverse mineral nitrogen dosages and various organic sources on the soil's available nitrogen levels (combined data over both seasons).

Where: B1: With bio fertilizer; **B2:** Without biofertilizer; **N1:**100% of NRD; **N2:**75% of NRD,**N3:** 50% of NRD; **N4:** 25% of NRD; **L1:** Control; **L2:** Compost tea**; L3:** Biogas and **L4:**Vermicompos

4. Discussion

Spinach yield and vegetative traits

The obtained results can be explained as follows; *Azospirillum brasilense* is known for its ability to fix atmospheric nitrogen and promote plant growth. When inoculated onto spinach plants, it colonizes the Rhizosphere and forms a symbiotic relationship with the plant roots. As a result, it enhances nitrogen availability to the plants, leading to improved growth parameters such as yield, plant height, and leaf number **(Galindo *et al.* 2022)**.

Nitrogen is a critical nutrient for plant growth and development. As the nitrogen recommended dose decreases, the availability of nitrogen to the plants diminishes, leading to decreased growth parameters. This is because nitrogen is a major component of chlorophyll, proteins, and nucleic acids, all of which are essential for plant growth. Insufficient nitrogen supply can limit photosynthesis, resulting in reduced biomass production (**Ahmed *et al.* 2020, Abd El-Hady et al. 2023)**.

Biogas, vermicompost extract, and compost tea are rich sources of organic matter and nutrients. When applied to the soil, they enhance soil fertility, improve soil structure, and provide essential nutrients to plants. Organic matter serves as a reservoir of nutrients, promotes microbial activity, and increases nutrient availability to plants over time. The effectiveness of these organic fertilizers in promoting plant growth is attributed to their ability to supply nutrients and improve soil health **(Yusof *et al.* 2018;Ai *et al.* 2020; Møller *et al.* 2022; Tikoria *et al.* 2022; Farid *et al.* 2023;Nada *et al.* 2023; Hafez *et al.* 2024)**. The combined treatment of biofertilizer, nitrogen, and liquid organic fertilizers showed synergistic effects on plant growth parameters. This could be due to the complementary actions of these treatments. Biofertilizers enhance nitrogen availability, while organic fertilizers improve soil fertility and nutrient uptake by plants. The combined effect of these treatments results in enhanced plant growth and productivity compared to individual treatments. Overall, the obtained results demonstrate the importance of biofertilization, nitrogen management, and organic fertilization in promoting sustainable agriculture by improving soil fertility, nutrient availability, and plant growth (Doklega and Abd El-Hady, 2017). Each treatment plays a specific role in enhancing plant growth parameters, ultimately leading to increased yield and productivity.

Leaves chemical constituents

The observed results can be explained by the distinct roles played by each treatment. Firstly, biofertilization with *Azospirillum brasilense* inoculation significantly enhanced the chemical composition of spinach leaves and nitrogen uptake, attributing to the bacterium's ability to fix atmospheric nitrogen and promote nutrient uptake by plants **(Zaheer *et al.* 2022)**. Conversely, decreasing nitrogen recommended doses led to diminished nutrient levels, highlighting the pivotal role of nitrogen availability in plant nutrition (**Ahmed *et al.* 2020)**. Among the liquid organic fertilizers, the superior performance of biogas, followed by vermicompost extract and compost tea, can be attributed to their rich nutrient content and soil conditioning properties, which enhance nutrient availability and uptake by plants **(Yusof *et al.* 2018;Ai *et al.* 2020; Møller *et al.* 2022; Tikoria *et al.* 2022; Farid *et al.* 2023;Nada *et al.* 2023; Abd ELhamied *et al.* 2024; Hafez *et al.* 2024)**. The interactive effects observed, particularly with the combined treatment **($B_1xN_1xL_3$),** signify synergistic interactions among biofertilizer, nitrogen doses, and organic sources, resulting in optimal nutrient uptake and plant nutrition. Notably, the presence of biofertilizer alongside any liquid organic fertilizer at reduced nitrogen doses demonstrated superior nutrient uptake compared to plants solely reliant on mineral fertilizers, underscoring the importance of integrating biofertilizers and organic sources to enhance nutrient availability and promote sustainable crop nutrition across multiple seasons**.**

Quality parameters (nitrate and nitrite)

The observed results can be explained by the specific roles of each treatment in influencing the concentrations of nitrate and nitrite in spinach plant leaves. Firstly, biofertilization treatments, such as the application of *Azospirillum brasilense*, likely contributed to the reduction in nitrate and nitrite accumulation in plant tissues **(Galindo *et al.* 2022)**. This reduction could be attributed to the bacterium's ability to enhance nitrogen uptake efficiency and promote nitrogen fixation, thereby reducing the need for excessive nitrogen fertilization and subsequently lowering nitrate and nitrite levels in the plant. Conversely, higher nitrogen-recommended doses led to increased accumulation of nitrate and nitrite, highlighting the direct relationship between nitrogen fertilization intensity and the accumulation of these nitrogen compounds in plant tissues. Additionally, the application of liquid organic fertilizers, particularly biogas, resulted in decreased nitrate and nitrite accumulation, possibly due to the organic matter's role in improving soil structure, enhancing nutrient availability, and promoting microbial activity, which collectively contribute to reduced nitrate and nitrite levels in plant tissues.

Postharvest soil analysis and total microbial count

The observed results can be explained by the distinct roles played by each treatment in influencing soil properties and microbial populations. Firstly, the combined application of biofertilizers and liquid organic fertilizers resulted in noticeable improvements in soil fertility parameters, particularly organic matter content, which is essential for sustaining microbial populations and promoting nutrient cycling. The utilization of biofertilizers, such as *Azospirillum brasilense,* likely contributed to the increase in total microbial count by enhancing nutrient availability and promoting microbial activity in the soil **(Rondina *et al.* 2020)**. Additionally, the application of liquid organic fertilizers, particularly biogas, vermicompost extract, and compost tea, further enhanced soil fertility and microbial populations through the addition of organic matter and beneficial microorganisms. The observed increase in available nitrogen levels with biofertilizer application underscores its role in nitrogen fixation and nutrient release in the soil, while the higher nitrogen doses also contributed to elevated nitrogen availability. Overall, these findings highlight the synergistic effects of biofertilizers and organic fertilizers in improving soil properties and microbial populations, ultimately promoting soil health and productivity across multiple seasons**.** The findings are in harmony with those of **Tikoria *et al.* (2022), Farid *et al.* (2023), Nada *et al.* (2023) and Hafez *et al.* (2024).**

5. Conclusion

The results highlight the positive impact of *Azospirillum brasilense* inoculation and liquid organic fertilizers on agronomic efficiency and nitrogen uptake in spinach plants, especially in enhancing shoot growth under low doses of mineral nitrogen. The presence of biofertilizer alongside any of the liquid organic fertilizers under 75% NRD resulted in higher values for yield and quality parameters of spinach compared to plants fertilized with 100% NRD without biofertilizer and liquid organic fertilizer.Therefore, integrating *Azospirillum brasilense* inoculation and liquid organic fertilizers such as biogas, vermicompost extract, or compost tea as alternatives to mineral nitrogen fertilization, particularly at lower NRD levels, is recommended to optimize spinach production while preserving soil fertility.

Given their low economic cost, ease of application, and consistent positive response across different nitrogen application levels, Azospirillum brasilense inoculation and liquid organic fertilizers emerge as a crucial technology for improving plant-soil nitrogen management and promoting sustainable spinach production in Egyptian conditions.

Conflicts of interest

The authors have declared that no competing interests exist.

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